REVIEWS

Resistance to antibiotics targeted to the bacterial cell wall

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Abstract: Peptidoglycan is the main component of the bacterial cell wall. It is a complex, three-dimensional mesh that surrounds the entire cell and is composed of strands of alternating glycan units crosslinked by short peptides. Its biosynthetic machinery has been, for the past five decades, a preferred target for the discovery of antibacterials. Synthesis of the peptidoglycan occurs sequentially within three cellular compartments (cytoplasm, membrane, and periplasm), and inhibitors of proteins that catalyze each stage have been identified, although not all are applicable for clinical use. A number of these antimicrobials, however, have been rendered inactive by resistance mechanisms. The employment of structural biology techniques has been instrumental in the understanding of such processes, as well as the development of strategies to overcome them. This review provides an overview of resistance mechanisms developed toward antibiotics that target bacterial cell wall precursors and its biosynthetic machinery. Strategies toward the development of novel inhibitors that could overcome resistance are also discussed.

Keywords: antibiotic resistance; bacterial cell wall; β-lactamases; Penicillin-Binding Proteins; fosfomycin; cycloserine; lipid II

Introduction

Peptidoglycan plays key roles in maintaining cell shape, providing an attachment site for surface-exposed virulence factors, and avoiding modifications in internal osmotic pressure.[1] In rod-shaped cells, such as *Escherichia coli*, its biosynthesis can be described in two phases: elongation, when the lateral cell wall is formed, and division, leading to the generation of daughter cells.[2] A common pool of precursors, synthesized in the cytoplasm mostly through the action of Mur enzymes, is required for both phases of peptidoglycan formation.[3]

The generation of the initial precursor, UDP-MurNAc, is catalyzed by the consecutive actions of MurA and MurB. MurA transfers enolpyruvate from phosphoenol pyruvate (PEP) to UDP-GlcNAc, thus forming UDP-GlcNAc-enolpyruvate (Fig. 1). As is the case for most enzymes involved in peptidoglycan biosynthesis, MurA is highly conserved among

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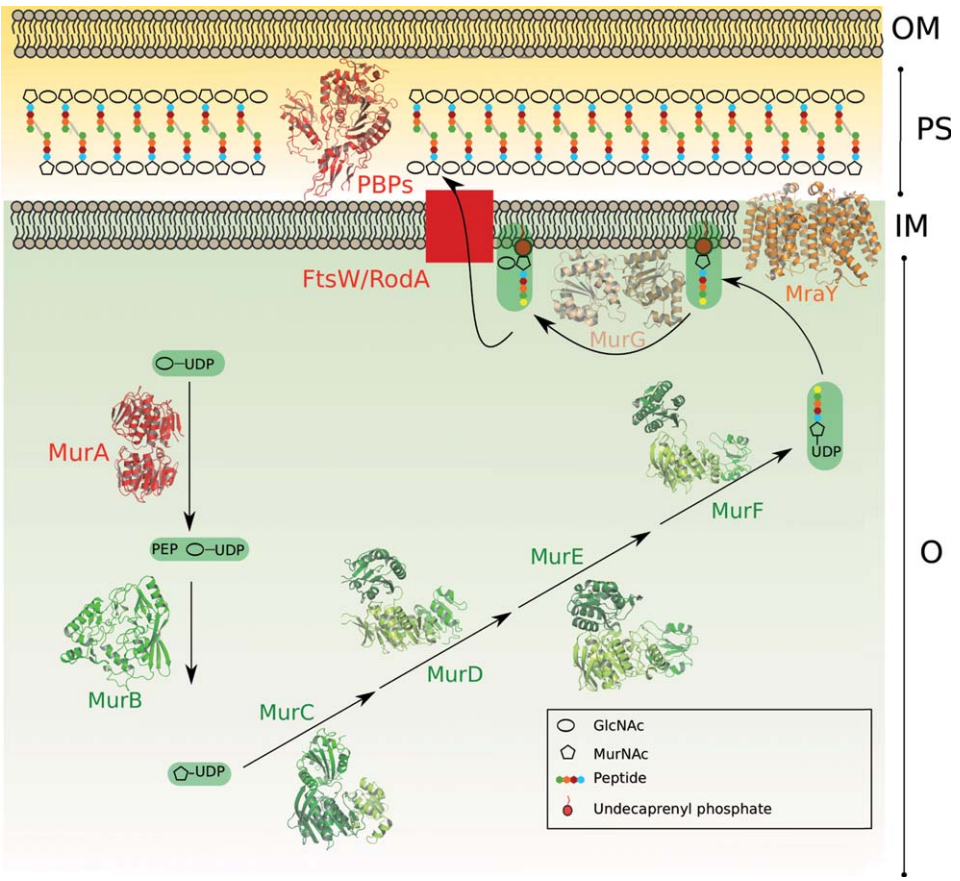

Figure 1. Schematic diagram of the cytoplasmic and membrane steps of the peptidoglycan biosynthetic pathway. The different domains of Mur enzymes are shown in shades of *green*. MurA and PBPs, which are the targets of antibiotics currently employed in hospital settings, are highlighted in *red*. OM, outer membrane; PS, periplasm; IM, inner membrane; C, cytoplasm.

bacteria, is essential for cell survival, and has no human homolog. MurA is the target of fosfomycin, an antibiotic currently in clinical use for which bacterial resistance is well studied (see below). MurB subsequently reduces UDP-GlcNAc-enolpyruvate to UDP-MurNAc;[3] although MurB has been extensively characterized, inhibitors for this enzyme are yet to be validated.[4] Subsequently, the ATP-dependent Mur ligases MurC, MurD, MurE, and MurF, sequentially link five amino acid residues to UDP-MurNAc, forming the UDP-MurNAc-pentapeptide. These enzymes are structurally and functionally well known and have been the targets of great interest in the rational search for antibacterials, notably through the employment of high-throughput screens. The fact that they share similar active sites makes them attractive targets for multitargeted inhibitors, which could potentially reduce the likelihood of mutational resistance.[5,6] However, and despite great efforts toward the identification of compounds with antibacterial activity, there are no antibiotics currently in use that target any of the Mur ligases.

MurF is the only Mur ligase which does not employ a single amino acid as substrate, but rather a dipeptide, D-Ala: D-Ala. Its synthesis requires racemization of L-Ala by the alanine racemase Alr, and subsequent condensation of two D-Ala molecules by Ddl, an alanine ligase.[3] Both Alr and Ddl are targeted by the antibiotic D-cycloserine, used as a second-line therapeutic in the treatment of multidrug resistant tuberculosis.[7]

Membrane-linked steps include the transfer of the MurNAc-pentapeptide moiety to the undecaprenyl phosphate lipid carrier by MraY, an integral membrane protein whose crystal structure has recently been solved.[8] This reaction generates Lipid I, to which MurG adds a GlcNAc moiety, resulting in Lipid II, the basic unit of the peptidoglycan polymer.[9] Despite the fact that MraY is an essential enzyme and a known target of natural antibiotics, several of which present considerable antibacterial activity, no MraY inhibitors are presently used in the clinic.[10]

The translocation of Lipid II from cytoplasm to periplasm is mediated by flippases. This step requires the activity of proteins of the SEDS family (shape, elongation, division, sporulation), such as FtsW, RodA, and SpoVE,[11] which have been shown to be essential in both Gram-negative and Gram-positive species.[12,13] To date, no specific ligands have

been developed to block their function. This is not the case for periplasmic proteins, such as Penicillin-Binding Proteins (PBPs), the macromolecular targets of β-lactam antibiotics. PBPs catalyze both the polymerization of Lipid II glycan chains and/or the crosslinking of stem peptides. It is the latter function, which recognizes the D-Ala:D-Ala moiety of the peptide, that is targeted by penicillin and its structural analogs.[14] PBPs are accessible (since they are located outside of the cell membrane), metabolize molecules that do not exist in eukaryotes (amino acids with D-chirality), and have no mammalian homologs, and thus have been extensively studied through biochemical, structural, and microbiological techniques.[15,16] Due to their extended employment in the clinic, different resistance mechanisms have arisen to counteract the targeting of PBPs by β-lactam antibiotics, underlining the importance for the search of novel molecules that do not carry the β-lactam ring.[17]

Despite the fact that the appearance of β-lactam resistant species has had dire consequences for the treatment of infections worldwide, the development of resistance has not been limited to this peptidoglycan biosynthesis step, and a number of other vastly employed antibiotics are now the targets of resistance mechanisms. This review aims at analyzing mechanisms developed by bacteria to inactivate or circumvent the action of drugs that target cell wall biosynthesis, with a main focus on the structural biology efforts that have been crucial toward the comprehension of such strategies. Novel molecules developed to counteract the action of resistant microbes are also highlighted.

Resistance to Antibiotics that Target Cytoplasmic Steps

Fosfomycin

Fosfomycin is a natural antibacterial produced by various *Streptomyces* and *Pseudomonas* species[18,19] and is the only antibiotic currently in clinical use that targets a Mur enzyme; its broad-spectrum characteristics allow it to be employed against both Gram-positive and Gram-negative bacteria. This PEP mimetic (Fig. 2) irreversibly inhibits MurA by alkylating the highly conserved catalytic cysteine, in a step that is facilitated by the initial binding of UDP-GlcNAc to the "open" form of MurA.[20] The resulting covalent adduct blocks catalysis, thus reducing the pool of peptidoglycan precursors. Crystal structures of multiple MurA–ligand complexes suggest that the mechanism of inhibition involves flexibility of a loop that lies in close proximity to the active site Cys residue, which can "trap" fosfomycin within the active site cleft.[20–22]

Interestingly, fosfomycin is a true textbook case involving a wide range of resistance mechanisms (Fig. 2), which include target modification, expression of antibiotic-degrading enzymes, reduced uptake, and rescue of the UDP-MurNAc biogenesis pathway. Resistance through modification of the catalytic site is naturally observed in fosfomycin-resistant species such as *Mycobacterium tuberculosis*, *Chlamydia thrachomatix*, and *Borrelia burgdorferi*,[23–25] since their MurA homologs carry a Cys–Asp mutation that prevents alkylation by the antibiotic. Amino acid substitutions that are distant from the catalytic site have also been identified in *E. coli* clinical isolates and were shown to confer additional resistance to fosfomycin.[26]

FosA, FosB, and FosX, all inactivate fosfomycin through direct modification of its chemical structure. The thiol transferases FosA and FosB and the hydrolase FosX catalyze the opening of the epoxide ring of the antibiotic.[27] FosA adds glutathione (GSH) directly to the oxirane ring of fosfomycin, generating an inactive form.[28] Similarly, in Gram-positive species that do not produce GSH, such as *Staphyloccocus aureus*, FosB adds bacillithiol (BSH, a low-molecular mass thiol) or L-Cys to fosfomycin.[29–31] The crystal structures of FosA and FosX, which display a large degree of structural similarity, have similar βαββββ motifs that harbor a metal-binding site within a cupped region. Notably, structures with bound product reveal that substrate binding is highly dependent on interaction with the Mn(II) center, and it is of interest that the metal itself has been proposed to play the role of acid catalyst in the fosfomycin hydrolysis reaction.[27,28,31,32] In addition to FosA/FosB/FosX, a second group of enzymes was also shown to confer intrinsic resistance in fosfomycin-producing species. FomA, FomB, and FosC display sequence similarities to eukaryotic protein kinases and catalyze the phosphorylation of the antibiotic through an ATP and Mg^{++}-dependent mechanism (Fig. 2).[33–35] FomA is an open αβα sandwich, and in the structure with bound fosfomycin, the antibiotic is shown to interact with the active site through its phosphonate group. A flexible "lid" region was suggested to become structured upon antibiotic binding, thus promoting its optimal positioning for catalysis.[34]

Due to its hydrophobic nature, fosfomycin is imported through the inner bacterial membrane via two nutrient membrane transporters: the glycerol-3-phosphate transporter GlpT and the glucose-6-phosphate transporter UhpT (Fig. 2).[36,37] Decreased expression or introduction of mutations in GlpT or UhpT can reduce fosfomycin uptake[26] resulting in lower susceptibility.[30,36] Most often, the defect in fosfomycin import is a consequence of complete deletion or a mutation resulting in a truncated form of the transporter,[26] but it remains unclear whether single substitutions can lead to fosfomycin resistance as well. The structure of GlpT, which contains 12

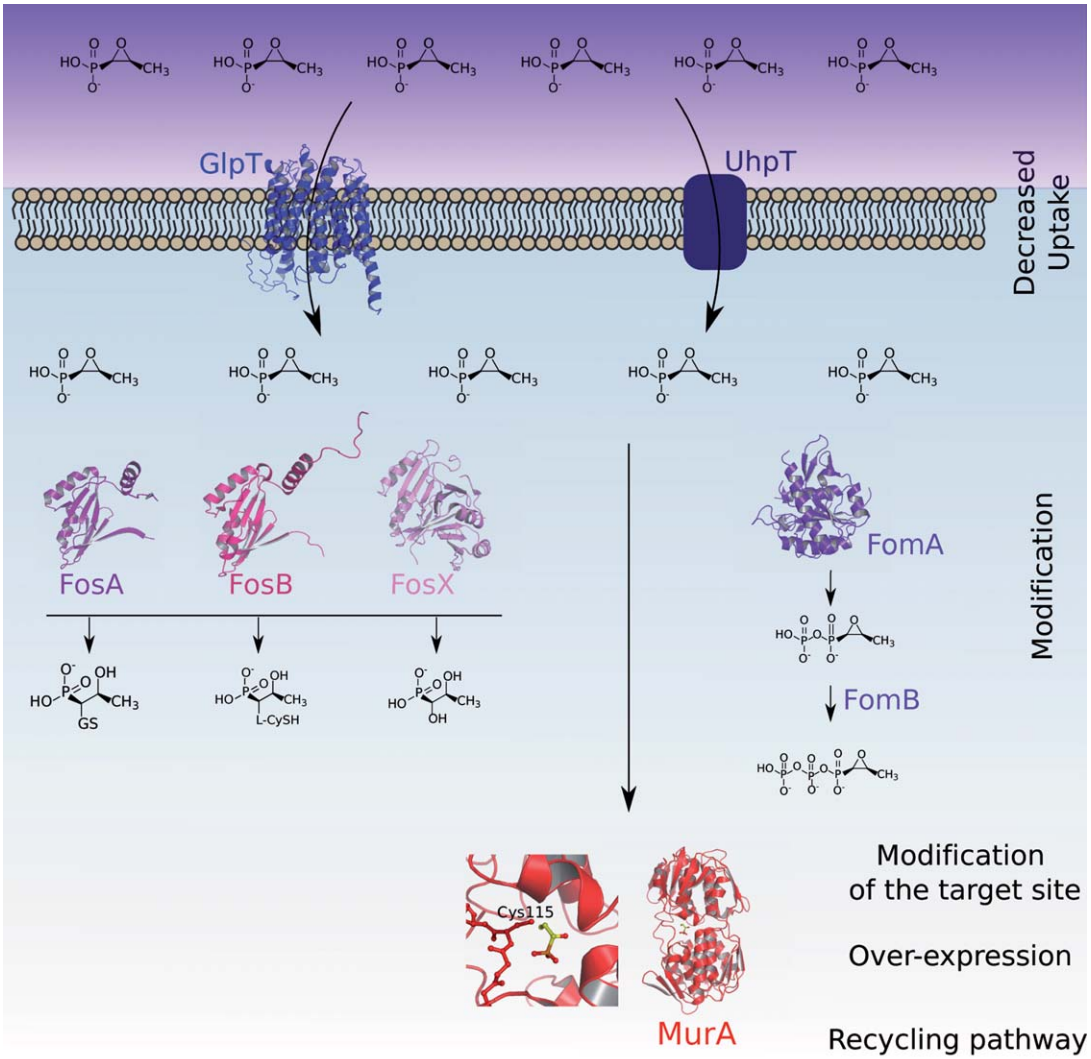

Figure 2. Mechanisms of fosfomycin resistance. Upon entry into the cell, fosfomycin can be phosphorylated by FomA/FomB, modified directly by FosA/FosB, or hydrolyzed by FosX. Other strategies include introduction of mutations within MurA, as well as its overexpression.

transmembrane segments, was solved to 3.3 Å. It is closed at the periplasmic face and displays an open pore at the cytoplasmic one; presumably, GlpT could display a "rocker-switch" motion which would allow substrate to be translocated through the center of the channel.[38] Despite the availability of this structural data, the precise mechanism of fosfomycin transfer through this transporter is still unclear.

Lastly, both transporters are known to be positively regulated by cyclic adenosine monophosphate (cAMP), and therefore lowering of intracellular cAMP concentrations through mutations in related genes can also induce resistance. This is the case for *cyaA*, which encodes adenyl cyclase, and *ptsI*, which is involved in the phosphoenolpyruvate:sugar phosphotransferase transport system.[26,30,39,40]

D-Cycloserine

D-cycloserine (seromycin), a cyclic structural analog of D-alanine, is a broad-spectrum antibiotic produced by some *Streptomyces* species.[3,41] Despite the fact that adverse neurological side effects limit its use in regular chemotherapy regimens, it is routinely employed as a second-line drug for the treatment of multidrug resistant *M. tuberculosis* infections.[42,43] D-cycloserine inhibits both Alr and Ddl.[3,43]

The major resistance mechanism involves the overexpression of AlrA.[44,45] AlrA is a two-domain molecule consisting of an α/β barrel in its N-terminal region and a C-terminal β-strand rich domain. The cofactor pyridoxal-5′-phosphate is covalently associated to a lysine residue within the active site, located in the N-terminal domain. In the structure of the cycloserine-bound form, it becomes evident that the antibiotic breaks the bond between PLP and lysine and forms an alternative covalent bond with the cofactor, thus becoming directly linked to the active site;[46] thus, overexpression of AlrA acts as a cycloserine "sink." Additionally, CycA, an importer of the amino acids β-/L-/D-alanine, glycine,

and D-serine, has also been linked to D-cycloserine uptake in *E. coli* and to the development of D-cycloserine resistance in mycobacterial BCG strains.[41,47] However, the above-mentioned mechanisms are not sufficient to fully explain D-cycloserine resistance, and it is believed that additional strategies could be involved.[47] In particular, mutations in a gene homologous to *E. coli* PBP4 were shown to confer resistance to D-cycloserine as well as to vancomycin in *Mycobacterium smegmatis*.[48]

The limited understanding of the resistance strategies toward D-cycloserine can be ascribed to the poor comprehension of the precise molecular mechanism of the drug itself. In particular, the prevalence of Alr or Ddl as the main target is still a matter of controversy, mostly due to the fact that both enzymes display complex regulatory mechanisms.[45] Moreover, Baisa et al. recently reported that a mutation in *dadA* is linked to resistance in *E. coli*, suggesting that an additional mechanism of resistance could involve an antagonizing effect of D-cycloserine on D-amino acid dehydrogenase (DadA) activity.[41]

Developing agents against cytoplasmic targets: Mur enzymes as a case study

Mur enzymes are attractive antibacterial development targets due to the fact that they are highly conserved, most of them are essential, they are specific to bacteria, and are well characterized both structurally and enzymatically. However, despite the extensive effort that has been dedicated to the search for inhibitors of Mur enzymes (A–G) that display antibacterial activity and that could eventually be pursued for employment in the clinic, apart from fosfomycin no Mur inhibitors are employed either in hospital settings or are within the antibiotic development pipeline.

A number of natural and synthetic MurA inhibitors were discovered in the past few years through structure–activity relationship experimentation and high-throughput screening efforts.[49] Interestingly, they present different modes of inhibition: covalent[50,51] and non-covalent[52] binding within the active site, or blocking of the transition from the open to the closed form, which is required for catalysis.[53,54] Although efficient *in vitro*, most compounds appeared to have no or weak antibacterial activity, and/or were not specific to MurA. It is of note that a number of preliminary "hits" were only superficially characterized, but would deserve further investigation toward lead optimization, keeping in mind the high-domain flexibility of the enzyme in structure-based drug design approaches.[49]

The search for MurB inhibitors has yielded a number of hits;[55–57] although none of the molecules could be shown to target MurB specifically *in vivo*, pyrazolidine analogs were reported to inhibit peptidoglycan biosynthesis.[58] However, UDP-MurNAc is a strong feedback inhibitor of MurA, and MurB inhibition could prevent this feedback process;[59] the consequent increase in the pool of UNAG-enolpyruvate produced by MurA could be competitive towards active site inhibitors of MurB.[4] This prompted the suggestion that dual inhibitors of MurA and MurB could be more suitable; substituted thiazolyl ureas and pyrazolidinediones have been found to inhibit both enzymes and to display some antibacterial activity[57,60] (but again there is no evidence that the inhibition of MurA/B is the only mechanism of action of these compounds). In addition, Kaur et al. recently identified three dual inhibitors of MurA and MurB from *Acinetobacter baumannii* using an *in silico* approach;[61] their results remain to be validated by enzymatic assays.

Mur ligases (Mur enzymes C–F) have been the subject of a very significant effort toward the development of inhibitors, a process that has been aided by the availability of structural data for all enzymes from different species. MurD, for example, has been particularly well characterized by high-resolution crystal structures in complex with phosphinate-, rhodanine-, D-Glu-, and thiazolidine-based inhibitors, some of which display weak antibacterial activity.[62–65]

Mur ligases are three-domain molecules (Fig. 1); the small N-terminal domain recognizes the peptidoglycan, the central domain binds nucleotide, and the C-terminal domain binds to the incoming amino acid.[5] This similarity is at the basis for the suggestion that a single compound could potentially inhibit all four ligases, thus preventing the development of drug resistance rapidly.[4] In support of this idea, several compounds that inhibit more than one Mur ligase have been identified.[63,66–68] To date, however, most of these compounds have shown little or no antibacterial activity. Notable exceptions are the MurF diarylquinolone inhibitors developed in the Bush lab, that generated an intracellular accumulation of UDP-MurNAc-tripeptide (and decrease of the pentapeptide) upon incubation with cells; however, the specific targeting of MurF within the cytoplasm was not shown.[69]

It is worthwhile mentioning that screens performed using industrial and commercial chemical libraries have had very limited success.[4,42,70] This result has been partly attributed to the unsuitable nature of the chemical libraries employed[71,72] but has also led to questions regarding the role played by the low permeability of the Gram-negative outer membrane in antibiotic intake, as well as the viability of Mur ligases as drug development targets.[4] It is of note that the interdomain conformational flexibility of the ligases could be a drawback for inhibition assays in which the enzymes may not be in the same conformation as they adopt *in vivo*, as well as for docking approaches that may not be representative of the conformations that enzymes adopt in the cell. In addition, Mur enzymes could be members of a multiprotein complex whose arrangement limits

the diffusion of inhibitors toward the active sites of the enzymes, thus providing a potential explanation as to why Mur ligases can be inhibited individually *in vitro* but not *in vivo*.[15,73,74] The exact reason for weak bactericidal activity of Mur ligase inhibitors remains to be elucidated. Work on coupling Mur inhibitors to a transport carrier or efflux inhibitor is also worth considering.[4,75,76]

Resistance to Molecules that Target the Periplasm and Membrane-bound Steps

Glycopeptides

The glycopeptides vancomycin, teicoplanin, and telavancin are currently employed in hospital settings as last resort antibiotics for the treatment of multidrug resistant infections of Gram-positive cocci, the latter only having been approved in 2009.[77,78] These molecules bind the D-Ala:D-Ala termini of peptidoglycan precursors, thus impeding proper transpeptidation and transglycosylation of peptidoglycan units.[79,80]

Resistance mechanisms associated with vancomycin, an antibiotic that has been employed for over 60 years, have been characterized in detail, and are mostly linked to the generation of alternate peptidoglycan precursors carrying D-Ala:D-Lac or D-Ala:D-Ser instead of D-Ala:D-Ala at their C-termini, for which vancomycin displays poor affinity.[81] Seven *van* gene clusters (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, and *vanL*) are involved in resistance development. Genes within these clusters encode dehydrogenases that generate D-Lac from pyruvate (or serine racemases that generate D-Ser), ATP-dependent ligases that catalyze the formation of D-Ala:D-Lac(D-Ser), and D-D-peptidases that hydrolyze the D-Ala:D-Ala moiety which is necessary for the constitutive expression of the unmodified peptidoglycan.[81,82]

The structures of a number of Van enzymes have been solved in the presence of substrates and inhibitors. VanA from *Enterococcus faecium* reveals a fold that is similar to that of D-Ala:D-Ala ligase B (DdlB);[83] notably, the recent structure of VanG, a D-Ala:D-Ser ligase, suggests that both VanA and VanG could have evolved from a common ancestral D-Ala:D-X ligase.[84] Recently, vancomycin resistance has been tackled from a different point of view, that of the structural characterization of an enzyme that is essential for conjugative transfer of plasmids that confer drug resistance. The crystal structure of the nicking enzyme in *S. aureus* (NES) in complex with DNA indicates binding grooves that could be targeted for the development of novel inhibitors that could prevent plasmid propagation, the earliest stage of the resistance process.[85]

New glycopeptide mimics against Gram-positive pathogens

A number of glycopeptidic agents are presently in different steps of the antibiotic development pipeline. Oritavancin (The Medicines Company) is a lipoglycopeptide which showed promising results in Phase III clinical trials; it inhibits peptidoglycan biosynthesis both by interacting directly with the stem peptide and with its pentaglycine bridge. In addition, a central hydrophobic group allows for interaction and disruption of the cell membrane, as is the case for telavancin.[86] This multiple mechanism of action confers activity against vancomycin-resistant organisms.[87,88] Dalbavancin (developed by Durata), which is very efficient against multidrug resistant *S. aureus* (MRSA) as well as vancomycin-resistant strains,[89] has completed Phase III clinical trials for skin infections. Both molecules still await FDA approval.[90]

β-Lactams

After its transfer to the periplasm, Lipid II is acted upon by PBPs, which catalyze the polymerization of the alternating MurNAc and GlcNAc chains (glycosyltransfer [GT]) and/or the crosslinking of the interchain stem peptides (transpeptidation [TP]). PBPs not only catalyze GT and TP reactions (activities addressed by class A and B high molecular mass enzymes), but low molecular mass PBPs are responsible for peptidic carboxypeptidation and endopeptidation, activities which regulate the level of crosslinking of stem peptides.[15,16,91] PBPs thus play a key role in the formation of the cell wall, and inhibition of the transpeptidase/carboxypeptidase reactions through the action of β-lactam antibiotics has been the reason for β-lactams being amongst the most widely used antibiotics worldwide. β-Lactams display a broad-spectrum of antibacterial activity and share a common core, the highly reactive four-membered β-lactam ring (Fig. 3), and function by structurally mimicking the D-Ala:D-Ala moiety of the stem peptide to form an irreversible penicilloyl-β-lactam intermediate within the active site of PBPs.[14] Blocking the transpeptidation reaction leads to weakening of the peptidoglycan and subsequent inhibition of cell growth or lysis. Since there seem to be at least one essential PBP in each bacterial species (in most cases, two), β-lactam use generally circumvents single mutation resistance mechanisms, requiring more complex strategies.[4,92]

There are four clinically important groups of β-lactam antibiotics: penicillins, carbapenems, monobactams, and cephalosporins (Fig. 3); a broad range of derivatives has been developed for all four classes.[93] The use of β-lactams has elicited the development of four major resistance mechanisms: (1) reduced membrane permeability or efflux increase through the action of multidrug efflux pumps; (2) expression of PBPs with reduced affinity to β-lactams or acquisition of "less sensitive variants"; (3) bypassing of the crosslinking step with L,D transpeptidases; (4) degradation of the antibiotic by

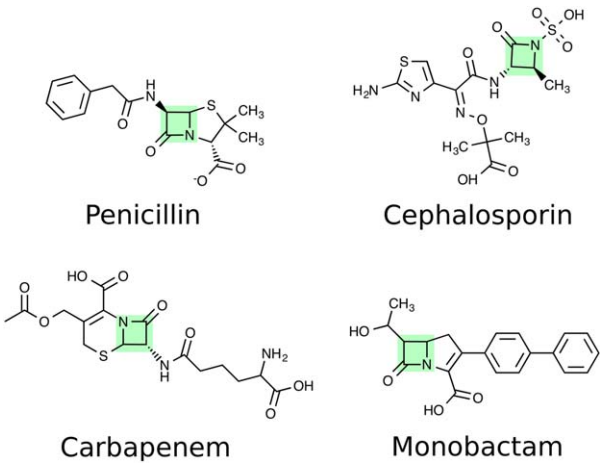

Figure 3. Chemical structures of the four clinically important β-lactam antibiotics. The common β-lactam ring is highlighted in *green*.

β-lactamases. The mechanisms that involve enzymes that are related to peptidoglycan biosynthesis (thus 2, 3, and 4) will be described in more detail here.

Altering the target: modified PBPs

PBPs from pathogens such as *Streptococcus pneumoniae* have been well studied from drug-sensitive and -resistant strains through the employment of techniques ranging from genetics to structural biology. This study has brought to light two major β-lactam resistance mechanisms involving PBPs. For example, PBP2x and PBP2b, both class B enzymes, are major determinants of resistance and strains carrying tens of mutations throughout the *pbp2x* and *pbp2b* genes have been identified in a number of clinical strains. This effect is generated by homologous recombination events between closely related species in environments where antibiotic pressure is high.[94–96]

Class B PBPs are modular proteins that display a membrane-anchoring region, an N-terminal pedestal, and a C-terminal TP domain. The TP domain displays a β-sheet fold packed by helices on both sides; this fold is similar in all structures of PBPs solved to date and is a clear signature of the protein family [Fig. 4(a)]. In PBPs involved in β-lactam resistance, mutations can be present throughout the entire protein sequence; however, they are notably concentrated around the active site region. Such mutations have been shown to induce modifications in active site geometry, by modifying the β3/β4 region [red in Fig. 4(b,c)].[97–99] Notably, a subtle modification in the position of β3 is also observed in the structure of PBP2a from *S. aureus*, an enzyme that is expressed in the case of an antibiotic challenge and that catalyzes stem peptide transpeptidation when the active site of the major class B PBP of the pathogen, PBP2, is inhibited by a β-lactam.[100] Interestingly, a reduction in active site accessibility of PBP5fm is at the basis for β-lactam resistance in the naturally resistant pathogen *E. faecium*.[101] It is of note that flexibility of the region in the vicinity of the active site has been recently suggested as playing a role the acylation reaction catalyzed by PBPs,[102–104] and induced-fit conformational modifications have been associated to antibiotic recognition events.[105]

The development of non-β-lactam inhibitors of PBPs has been a strategy of choice, with the goal of circumventing (at least temporarily) the resistance process. A number of molecules have been developed, notably lactivicins,[106,107] rhodanines,[108] quinolones,[109] and boronates.[110,111] Of these, lactivicins and boronates have been shown to be able to not only inhibit specific PBPs but also eliminate drug-resistant bacteria.[106,110] In addition, new fluorescence-based assays that can potentially be employed to test chemical libraries for PBP inhibitors are now available,[112,113] paving the road to the exciting possibility of the identification of novel TP active site inhibitors.

In some organisms, low-β-lactam affinity PBPs can only proceed with transpeptidation if the substrate (the stem peptide) is branched. This is the case for methicillin resistance mediated by PBP2a in *S. aureus*, and penicillin resistance mediated by PBP2x in *S. pneumoniae*.[114,115] The generation of crosslinks between adjacent stem peptides is catalyzed by nonribosomal peptidyl transferases that belong to the Fem protein family. Fem transferases transfer L-amino acids and Gly to peptidoglycan precursors directly from aminoacyl-tRNAs in a ribosome-independent manner, and have the ability to do so by employing either nucleotide precursors or lipid intermediates.[116] Members of this family include FemABX from *S. aureus*, MurMN from *S. pneumoniae*, BppAiA2 from *E. faecalis*, and the well studied FemX from *Weissella viridescens* ($FemX_{Wv}$).

In *S. aureus*, transposon-mediated mutagenesis identified factors essential for methicillin resistance (genes *fem*A and *femB*). The two genes were shown to be essential for the incorporation of glycine into pentaglycine cross-bridges, and introduction of mutations result in altered peptide bridges and an increase of susceptibility toward β-lactams.[117] Shortened pentaglycine interpeptides result in a failure in crosslinking, and thus in loss of methicillin resistance mediated by PBP2a, prompting the interest in the development of Fem inhibitors. Interestingly, peptidyl-RNA conjugates were shown to inhibit $FemX_{Wv}$ *in vitro*,[118] and an inhibitor of *S. aureus* FemA potentiated the activity of imipenem against MRSA strains,[119] indicating that it could be possible to develop inhibitors that could be employed in conjunction with β-lactams.

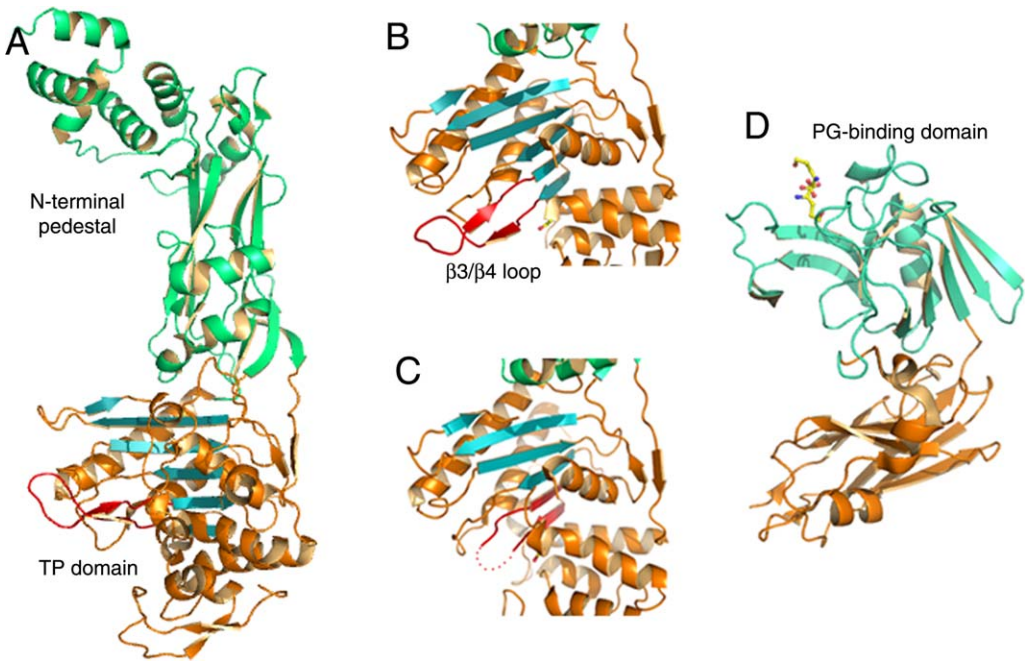

Figure 4. PBPs and L,D-transpeptidases recognize peptidoglycan and β-lactams through α/β folds. (**A**) PBP2b from *S. pneumoniae* folds into distinct domains, where the C-terminal, transpeptidase domain harbors the active site within an α/β fold, (**B**) Zoom of the β3/β4 region of PBP2b (indicated in *red*), which shows flexibility in a number of PBPs and peptidoglycan-recognizing enzymes. (**C**) Same region as in (B), but from PBP2b from a drug-resistant *S. pneumoniae* strain, indicating that the loop between β3/β4 could not be traced in the electron density map and is thus indicated with *dots*. (**D**) Structure of L,D transpeptidase from *M. tuberculosis* bound to a short region of a peptidoglycan substrate.

Bypassing a step in the pathway: L,D-transpeptidases (LDT)

Despite the fact that the transpeptidation reaction catalyzed by PBPs has been considered as being essential for peptidoglycan stability and bacterial survival, it can be bypassed by LDT, enzymes already identified in *Enterococcus*, *Mycobacterium*, and *Clostridium* spp. These enzymes employ a catalytic triad (His, Cys, Asp) in order to crosslink the third residues of neighboring stem peptides (3 → 3 bond), unlike PBPs, which catalyze a 4 → 3 bond. Notably, their differences also lie in the chirality of their substrates (L,D and D,D for L,D transpeptidases and PBPs, respectively).[116,120,121] The structures of LDTs from different species reveal a two-domain molecule [Fig. 4(d)], with the catalytic domain displaying a mobile loop element in the vicinity of the substrate-binding site,[122–124] which is reminiscent of PBPs, as mentioned above.

LDT production in *E. faecium* was shown to depend on activation of a D,D carboxypeptidase that generates tetrapeptides from the natural pentapeptides present in the peptidoglycan, thus providing the substrate for LDTs and asserting that they will catalyze all crosslinking reactions in these resistant species.[125] Bypassing the PBP catalysis step by LDTs results in a very high level of resistance to ampicillin, and moderate levels toward ceftriaxone, in a mechanism that involves direct acylation of the active site Cys.[126] Notably, in *M. tuberculosis*, LDT activity is the dominant strategy for peptidoglycan crosslinking during the chronic phases of infection, suggesting that a combination of β-lactams and LDT inhibitors could prove to be an excellent strategy for control of this pathogen.[120]

Destroying the antibiotic: β-lactamases

β-Lactamases hydrolyze the β-lactam ring of the antibiotic, thus inactivating it before it has the opportunity to block the PBP active site. They are the main antibiotic resistance mechanism in Gram-negative bacteria. To date, the Protein Data Bank includes more than 700 β-lactamase crystal structures, attesting to the importance of and interest in these enzymes. Upon expression, β-lactamases can have three main fates: secretion into the periplasm (in the case of Gram-negative organisms), association to the membrane, or secretion into the environment.[127] The structural similarities observed between β-lactamases and PBPs have led to the hypothesis that the former enzymes arose as *Streptomyces* spp and other soil microorganisms secreted soluble forms of PBPs as a primary defense mechanism against exposure to increasing concentrations of β-lactams.[128]

β-Lactamases have been historically classified into four classes (A to D) based on sequence

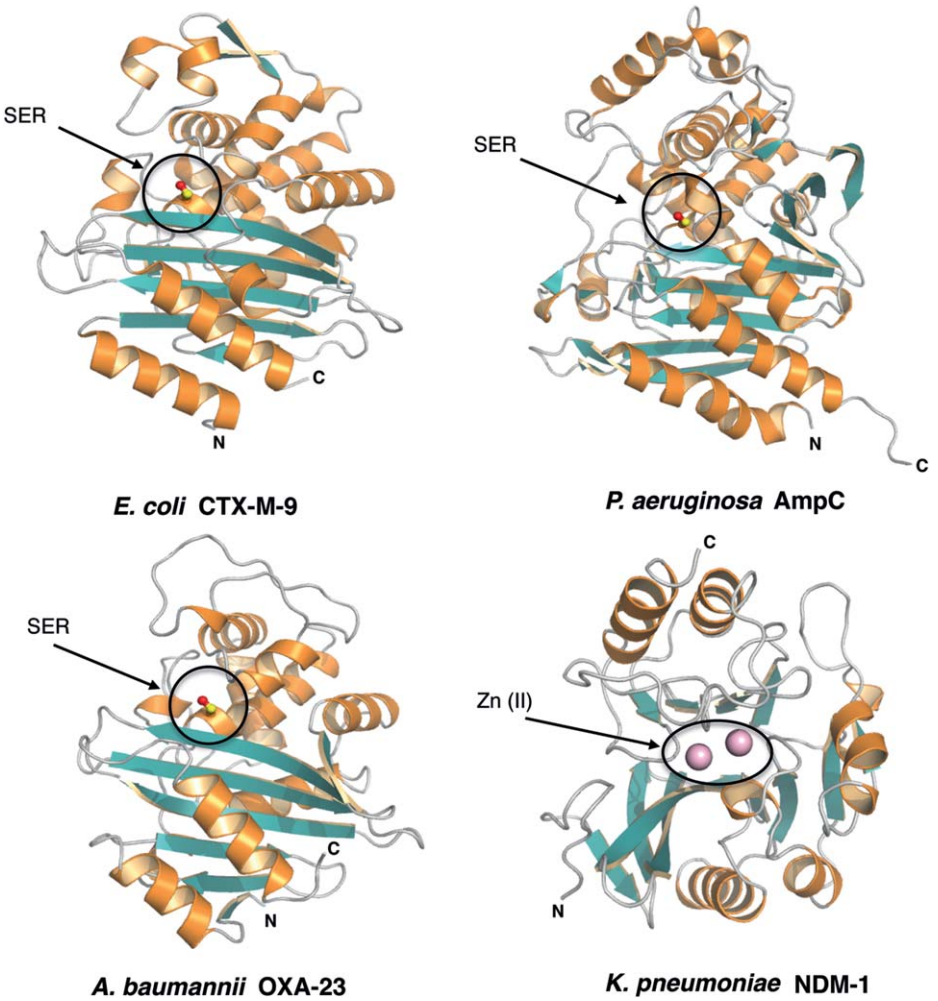

Figure 5. Structures of class A, C, D, and B β-lactamases. The conserved catalytic serine (SER) of classes A (CTX-M-9), C (AmpC), and D (OXA-23) β-lactamases is embedded at the interface between two closely interacting domains, shown in *orange* and *green*. NDM-1, a class B enzyme, differs from these serine-β-lactamases in fold and chemical mechanism. The enzyme shows a αβ/βα fold with an active site located at the edge of the β-sandwich. The active site is occupied by divalent zinc ions shown as *pink spheres*.

homologies.[129] Classes A, C, and D display similar folds and harbor an active site serine required for the formation of an acyl–enzyme complex with the incoming β-lactam, followed by hydrolysis of the intermediate.[130] Class B enzymes are metalloproteins that require one or two zinc ions to hydrolyze the β-lactam ring, and differ from the other classes in fold, sequence, and mechanistic details.[131] An updated classification of these enzymes also takes substrate and inhibitor specificities into consideration,[132] and ranks β-lactamases into group 1 (class C enzymes, including cephalosporinases); group 2 (classes A and D, including carbapenemases); and group 3, the metallo-β-lactamases.

Class A β-lactamases include penicillinases that are predominant in pathogens including staphylococci and enterococci. This class also includes the extended spectrum enzymes (ESBL). Classes A, C, and D β-lactamases share a common fold where the catalytic serine is embedded at the interface between two closely interacting domains, one of which is composed of a central β-strand surrounded by helices (and shows clear similarities to the TP domain of PBPs; green and orange, Fig. 5), and a second, mostly helical domain. TEM-1, and SHV-1 β-lactamases, clavulanate-resistant ESBLs, are frequently produced by clinical isolates, and have the ability to hydrolyze penicillins, monobactams, and a broad range of cephalosporins. The structures of several well-studied ESBLs, such as CTX-M, Toho-1, SHV-1, and KPC-2 have been solved in the presence of a number of ligands,[133–136] providing key information for the potential development of inhibitors.

Class C (group 1) β-lactamases include enzymes that have the ability to hydrolyze a broad range of β-lactam antibiotics, including third generation cephalosporins. AmpC β-lactamases are representative of this class, and their crystal structures reveal

a fold that is highly similar to that of class A enzymes, despite an active site pocket that is more open, resulting in their ability to better accommodate the hydrophobic moieties of cephalosporins.[137] AmpC β-lactamases are resistant to all known classes of inhibitors, with the exception of boronic acid analogs. They can be chromosomally located or transmitted through mobile genetic elements; the chromosomally encoded β-lactamases are either constitutively expressed or require the presence of a specific regulatory system for induction.[138] Strikingly, pathogens expressing inducible AmpC variants are of more acute clinical relevance due to the high-level expression of the enzyme upon exposure to β-lactams.[139] Notably, this process requires a complex regulatory mechanism that involves enzymes of the cell wall recycling pathway that will be described below.[140]

Class D enzymes such as oxacillinases display not only the ability to hydrolyze carbapenems but also isoxazoyl β-lactams such as methicillin and oxacillin (hence the name, oxacillinases, or OXA β-lactamases).[141] OXA-type β-lactamases are often encoded by genes located in integrons, but recently plasmid and transposons encoding OXA enzymes were reported in Gram-negative species.[142] OXA β-lactamases show little sequence similarity to other classes of β-lactamases. A characteristic feature of OXA enzymes is the conserved carboxylated lysine in the active site, which most likely serves as a general base that activates the serine nucleophile.[143,144] Furthermore, the high hydrophobic character and the larger size of the active site cleft of OXA-type enzymes frequently result in an extended spectrum of antimicrobial activity.[143] Notably, class D β-lactamases of *A. baumannii* and *Pseudomonas aeruginosa* are at the source of well-documented failures in clinical treatment strategies.[145]

Metallo-β-lactamases (MBLs, class B, group 3) contain either one or two zinc ions within the active site in order to catalyze hydrolysis of the β-lactam ring. MBLs display the characteristic αβ/βα fold, with the active site located within a shallow groove at the interface between the two domains.[146–149] These enzymes hydrolyze almost all known β-lactams with the exception of monobactams. Plasmid-encoded MBLs represent a major resistance mechanism of Gram-negative bacteria, including *P. aeruginosa*, *A. baumannii*, and enterobacteria.[150] Recently, a novel class B enzyme, the New Delhi metallo-β-lactamase (NDM-1), was isolated from a *Klebsiella pneumoniae* strain that that was resistant to all β-lactams, even late-generation carbapenems including meropenem and imipenem.[151] Since then, the number of cases of NDM-producing pathogens has increased drastically worldwide, and the term "superbug" was coined to indicate NDM-expressing bacteria.[131,152] Strikingly, plasmids encoding NDM-1 often co-harbor genes encoding proteins involved in mechanisms of resistance to agents other than β-lactams, thus exacerbating the possibility of treatment failure.[150]

The crystal structure of NDM-1 reveals notable structural similarities to other MBLs[153–155] (Fig. 5). The active site is located at the bottom of a shallow, hydrophobic pocket that is enlarged in respect to that of other MBLs, an observation which could potentially be at the basis of the broad substrate selectivity of this enzyme. The catalytic activity of NDM-1 also depends on the presence of two zinc ions that are located within different binding environments; Zn^{2+}(I) is coordinated by three conserved histidine residues, while Zn^{2+}(II) is ligated by an Asp–His–Cys triad. Catalytic activity requires mobility of two loops that are located in close vicinity to the active site; their flexibility has been linked to optimal substrate hydrolysis.[156] Notably, the search for NDM inhibitors has led to the identification of natural compounds that bind to NDM-1 with high affinity, and could be eventually explored as leads.[157]

Mechanisms of inducible β-lactamases

Many bacteria induce β-lactamase expressing genes in the presence of high levels of antibiotics, and this phenomenon is now known to be tightly linked with the process of peptidoglycan precursor recycling. In Gram-negative organisms, two major mechanisms have been well characterized: the AmpG–AmpR–AmpC pathway, and the BlrA/BlrB two component regulatory system (Fig. 6).

Regulation of AmpC depends on the relative concentrations of cytoplasmic anhydromuropeptides. In the absence of β-lactam pressure, UDP-MurNAc-pentapeptide is bound to the transcriptional regulator AmpR, which inhibits expression of AmpC.[138] Upon inhibition of PBPs by β-lactams, however, peptidoglycan biosynthesis is slowed down or blocked, while the activity of autolysins remains constant, resulting in accumulation of anhydromuropeptides in the periplasm. Muropeptides enter the cytoplasm through the AmpG permease, and their GlcNAc moiety is hydrolyzed by NagZ.[158,159] The accumulation of anhydromuramyl peptides in the cytoplasm results in the displacement of UDP-MurNAc-pentapeptide from AmpR. AmpC is thus expressed and subsequently secreted to the periplasm, where it hydrolyzes the β-lactam ring of the antibiotic.[138]

A different regulatory mechanism was identified in bacterial species of the genus *Aeromonas,* which control the expression of AmpC using a two component regulatory system consisting of the sensor kinase BlrB and the response regulator BlrA (Fig. 6). Both proteins are closely related to the *E. coli* CreBC two-component regulatory system, which is involved in the regulation of key metabolic pathways in response to nutrient deprivation.[160] The inhibition of the TP activity of PBPs by β-lactams results in the accumulation

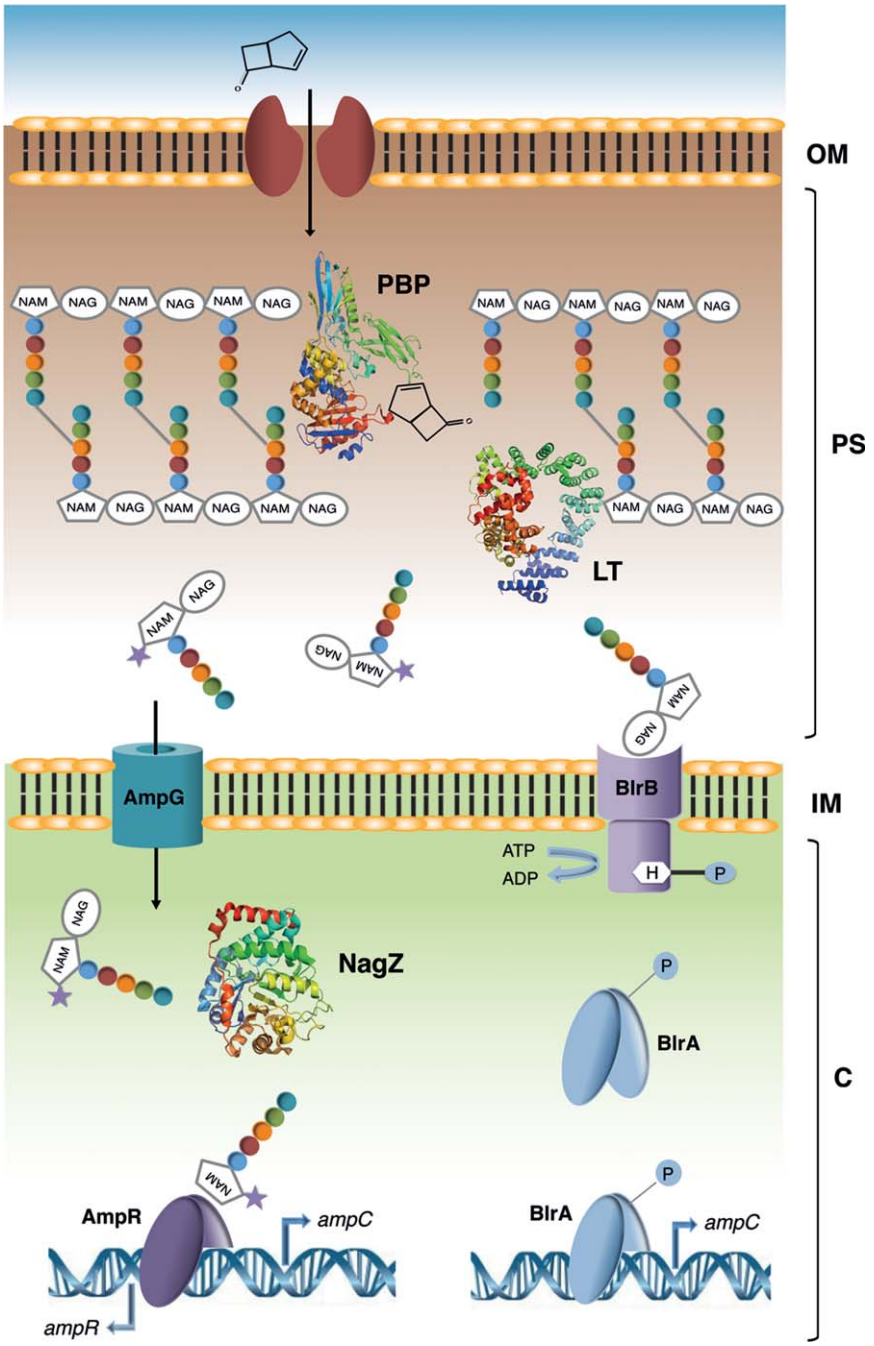

Figure 6. Schematic model of AmpC β-lactamase induction in Gram-negative organisms. The AmpG–AmpR–AmpC pathway as well as the BlrA/BlrB two component regulatory system are indicated. The presence of β-lactams results in excessive breakdown of the murein sacculus and thus in accumulation of muropeptides. This accumulation causes either the activation of AmpR (AmpG–AmpR–AmpC pathway shown on the **left**) or the phosphorylation of BlrA (BlrA/BlrB two component regulatory system shown on the **right**); in both situations, there is induction of the *ampC* gene. LT, lytic transglycosylase; PBP, Penicillin-Binding Protein.

of disaccharide pentapeptides in the periplasm, inducing autophosphorylation of the kinase domain of BlrB. Transfer of the phosphate moiety to BlrA causes binding of the response regulator to the promotor region upstream of the genes that code for the Amp, Cep, and Imi β-lactamases, inducing expression.[161]

Despite the fact that both mechanism of induction display significant differences, both pathways seem to be induced by an intracellular increase of muropeptides and are thus linked to cell wall recycling. Consequently, enzymes involved in the β-lactamase induction pathway represent an attractive target for the development of effective inhibitors, which could facilitate therapeutic treatment in combination with classical β-lactams.

This is the case of the transmembrane protein AmpG. Inactivation of AmpG fully restored β-lactam

susceptibility in resistant strains of *P. aeruginosa*, even minimizing the effect of an antibiotic efflux pump.[162] Furthermore, employment of the AmpG inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) decreased the expression of AmpC in *P. aeruginosa* strains; combination of the inhibitor with β-lactams improved the MIC for specific pseudomonal strains.[163] Interestingly, inhibition of the glucosaminidase NagZ also caused a 75% reduction in AmpC expression and an increase in β-lactam susceptibility in *E. coli* strains.[158] The crystal structure of NagZ in complex with an inhibitor reveals that the molecule binds to the center of NagZ's TIM barrel. This high-resolution structure was essential for the development of novel glucose analogs with even higher selectivity for NagZ that also showed attenuation of AmpC expression in *E. coli* strains.[164]

Lastly, the transcriptional regulator AmpR could also be considered as a potential target for novel inhibitor development. Binding of the activator ligand to the active site of AmpR generates a conformational change leading to de-repression of the regulator. A potential approach for the development of inhibitors against AmpR would involve the identification of small molecules which bind with high affinity to its active site, causing the regulator to remain in its repressed state, resulting thus in steady inhibition of β-lactamase expression.[140] The ubiquitous presence of proteins involved in regulation of peptidoglycan recycling in bacterial genomes suggests that the development of inhibitors of these enzymes could prove to be useful for combination therapy strategies.

Fighting β-lactamases

Although most β-lactam antibiotics are susceptible of being hydrolyzed by a subset of the 1300 β-lactamases that have been identified, new molecules that are able to at least partially circumvent this effect are being presently developed. BLA30072 (Basilea), a monocyclic β-lactam currently undergoing phase I clinical trials, is resistant to hydrolysis by MBLs, binds to distinct PBPs, and can kill *P. aeruginosa* and *Acinetobacter* that secrete these enzymes.[165] In addition, combinations of different inhibitors are promising strategies. Ceftolozane is a cephalosporin that on its own is susceptible to extended spectrum β-lactamases, but when employed in addition to tazobactam is efficient against *E. coli* and *P. aeruginosa*.[166] A number of other combinations are presently being explored, and it is likely that combination therapy will provide a solid approach for the development of anti-infectives, especially against Gram-negative pathogens.[90]

Conclusion

Bacteria have developed a number of resistance mechanisms to circumvent the targeting of its Achilles heel, the peptidoglycan biosynthetic machinery. However, the past few years have seen a substantial increase in the knowledge regarding not only the mechanism of action of cell wall-targeting antibiotics, but also in the development of new inhibitors that could target these resistance strategies. Structural biology will continue to play a critical role in the search for novel combination therapies that circumvent such resistance processes.

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